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A study of the mechanism of rubidium accumulation in Euglena gracillis.

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A STUDY OF THE MECHANISM OF RUBIDIUM ACCUMULATION IN *EUGLENA GRACILLIS*

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INTRODUCTION

For many years, plant physiologists have been carefully studying the basic nutritional requirements essential for optimum plant growth. Concomitant with the problem of which nutrients to use and their apparent concentrations, is the even more basic problem of how such material is transported across the cell membrane. If we fully understood the mechanism of transport, we might better be able to control this phenomenon to benefit plant growth.

Plant physiologists originally thought that salts in the soil simply entered the roots by mass flow or by diffusion. Recognizing that plants often contained very different concentrations of salts than found in their growth medium, it was suggested that the process of salt entry into the plant was more complicated than a simple process like diffusion. Gradually, within the past thirty years, an hypothesis has evolved suggesting that some type of carrier is selectively transporting ions across the plant cell membrane. In 1960, Sutcliffe (56) published his now classic paper demonstrating that the carrier mechanism for ion transport is proteinacious; this finding has opened a whole new area of investigation for the plant nutritionist. It is the aim of this thesis to further investigate the possibility of a protein functioning in ion transport.

LITERATURE REVIEW

Mechanism of Ion Transport in Plants

During the past half century many theories have been presented attempting to explain the mechanism of ion uptake by plant cells. Several excellent reviews of this subject are now available by Sutcliffe (57, 58), Steward and Sutcliffe (52), Russell (44), Jennings (25), Wallace (61), Jyung and Wittwer (26), and many others.

In a recent review on ion transport, Brouwer (6) has classified ion absorbtion into three parts: a) ions held in water free space, b) ions retained in Donnan free space, c) ions which are held after the above two groups are removed.

Water free space in a plant cell is that area of the cell which is rapidly penetrated by ions from the medium in which the cell is located. Usually the internal ion concentration is the same as the external solution and can be easily removed by washing with distilled water. Donnan free space is similar to water free space except that ions are retained by electrostatic charges. Ions in this latter group can be removed by equivalent exchange with other ions located in the outer medium.

The actual site of the above-mentioned fractions is presently a highly debated question. Brouwer (6), for example, suggests that two types of free space are located in the cell wall and outer cytoplasm. This suggestion could indicate that the permeation barrier would lie either in the

tonoplast or the inner part of the cytoplasm. Jennings (25) devotes considerable time to discussing the various possibilities for the location of the permeation barrier. Although this problem is fascinating, it is not the major concern of this paper.

There are two postulated types of ion uptake for most plant cells: passive uptake and active uptake. Passive uptake is mainly the absorption of material by physical means. Active uptake is completely dependent upon metabolic energy.

Passive uptake can be subdivided into several classifications: diffusion, the dispersion of one constituent randomly within that of another; mass flow, the passage of a solute by some force like that of gravity; ion exchange, the diffusion of ions across a membrane in order to establish an equilibrium on either side; Donnan equilibrium, a more complex form of ion exchange where one or more ions are electrochemically bound to the exchange system while allowing other ions to move freely throughout the system; and adsorption, the binding of material onto the outer surface of the cell wall. Any or all of these types of passive uptake might be operating at the same time within the cell. All of the passive uptake mechanisms are only slightly affected by changing the temperature, for they have a Q_{10} of about 1.2 (57).

Although active uptake is known to require metabolic energy, the actual mechanism of transport is still uncertain. Sutcliffe (57) reviews in great detail many of the possible hypotheses that could explain the active uptake mechanism.

Lundegårdh (31) in 1939 proposed that salt or anion respiration (respiration stimulated by the addition of a salt, particularly the anion) was directly related to the cytochrome system. This hypothesis suggested that anion absorption was mediated by electron transfer of the cytochrome system and cations follow the anions passively to maintain electrical neutrality. Sutcliffe (57), Jennings (25), Street (54), and Russell (43) all give excellent reasons why Lundengardh's theory cannot be the actual mechanism. They find his theory does not explain the following: a) not all anions compete for the same carrier; b) evidence has been reported that the rate of anion transport has exceeded the theoretical rate of anion uptake in relation to respiration; c) uptake can still proceed on the stored products of respiration, even if respiration has stopped; d) cation absorption seems to occur quite independently of anion absorption; and e) many plants are highly selective for different cations.

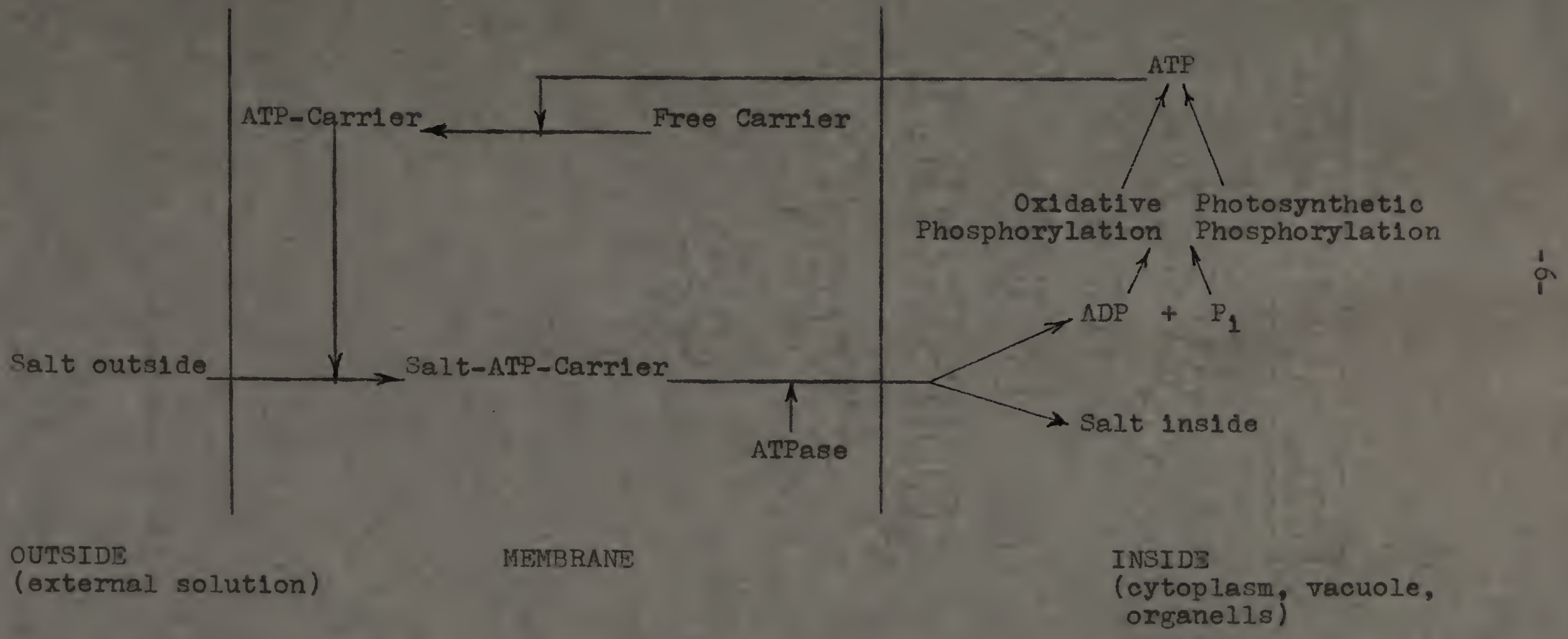
Most of the workers in ion transport today envision a carrier mechanism that serves to move ions across the permeation barrier. Sutcliffe (57) describes the mode of action of a carrier as starting with the synthesis of the carrier molecule which then forms a complex with the ion on the outer membrane surface. The carrier complex then transports the ion across the membrane where the ion would then be released. The free carrier would finally be returned to the outer surface of the membrane. Jyung and Wittwer (26) elaborated on

the pathway by implicating the use of adenosine triphosphate (ATP) as an energy source that would thereby activate the carrier (see Figure 1).

The actual nature of the carrier is still uncertain. Steward and Street (51) in 1947 suggested that a protein precursor serve as an amphoteric carrier for both anions and cations. Bennet-Clark (2), in 1956, offered the possibility that the carrier could be lecithin and that the phosphate group would bind cations, while the choline group would bind anions. Once the complex was transferred into the internal side of the membrane, the enzyme lecithinase would split the molecule into choline and phosphatidic acid, thereby releasing the bound ions. ATP, choline acetylase, and choline esterase would help regenerate new lecithin. Unfortunately, little experimental evidence is available to support or refute this theory.

Sutcliffe (56), in 1960, greatly advanced the carrier theory by retarding Na^+ accumulation during a 24 hour experimental period in red beet and carrot sections using the potent specific inhibitor of protein synthesis, chloramphenicol at 2 mg/ml (6 mM). Uhler and Russell (59) pretreated barley seedlings for 24 hours with 1 mg/ml chloramphenicol which reduced the uptake of Ca^+ and Rb^+ during the following 24 hours. Determinations of protein synthesis were not reported in these papers. Results obtained by Peaud-Lenoel and De Gournay-Margerie (37) indicated that glucose uptake

Figure 1. Theoretical Carrier System for Ions (26).



was inhibited 24.5 per cent within 1 hour when pretreated with the antibiotic for 30 minutes. Chloramphenicol at 0.33 mg/ml had practically no effect on glucose uptake but at 1 mg/ml reduced by almost 50 per cent the amount of ^{35}S-sulfate incorporation into protein. Total $SO_4^=$ uptake was also reduced. Since the total amount of $SO_4^=$ taken up was reduced, it is difficult to determine the extent of inhibition of protein synthesis from inhibition of uptake of the $SO_4^=$. Bowling (4) completely inhibited the uptake of K^+ into intact castor bean plants in 2 hours by using 2 mg/ml chloramphenicol. It should be noted that Bowling did not actually measure the amount of K^+ that entered the plant but, rather, he determined the amount of K^+ left in the solution after the experimental period.

Ellis (10) reported that using 1.7 mg/ml chloramphenicol inhibited ^{14}C-labeled glycine incorporation into the trichloroacetic acid (TCA) insoluble residue of beet slices after a 2 hour experimental time. However, the chloramphenicol did not reduce the incorporation of labeled leucine or threonine into the same type of residue. Ellis has questioned whether or not the chloramphenicol really was inhibiting protein synthesis even though salt uptake was reduced (10). He also used the L-isomer of chloramphenicol (9) and was able to reduce $SO_4^=$ uptake. Ellis further questioned the action of chloramphenicol because this isomer does not inhibit protein synthesis in bacteria.

Jyung et al. (27) in 1965 treated enzymatically-isolated tobacco leaf cells with 0.25 mg/ml D-isomer of chloramphenicol for 12 hours and found 84 per cent inhibition of ^{86}Rb accumulation and 71.3 per cent inhibition of ^{14}C-l-glycine incorporation into protein. When they used the L-isomer of chloramphenicol, they found 65 per cent and 48 per cent inhibition respectively.

None of the above workers found chloramphenicol to have any effect on respiration.

Jacoby and Sutcliffe (23) found that by treating carrot root slices with KNO_3 for 96 hours, the rate of protein synthesis tripled while K^+ uptake increased only 1.2 times. Chloramphenicol at 2 mg/ml in the presence of KNO_3 inhibited both systems only 50 per cent. Their conclusion was that K^+ transport was related to a particular type of protein which is probably located on the surface of the cell membrane. For this reason they suggest that it might be difficult to find a definite relationship between protein synthesis and ion uptake.

Sutcliffe has recently mentioned the possibility that micropinocytosis might be working as part of the carrier mechanism in plants. Pinocytosis is known to be involved with the ingestion of solid material into amoeba cells (21). He suggests that it is possible that ions would bind to certain sites on the membrane wall, such as proteins, and that a micro-invagination (micro-pinocytosis) would then move the bound ions within the permeation membrane in a micro-vesicle. Once

inside of the membrane, the ions could be released by the enzymatic breakdown of the micro-vesicle membrane. This mechanism might help explain how some electrically neutral molecules, like glucose, are actively transported into the plant cell.

Other workers have spent considerable time in an effort to determine how many carriers actually are at work in plants. Epstein et al. (12, 13, 42) report the existance of two carriers for K^+ in barley roots. The first carrier has a high affinity for K^+ when K^+ is less than 1 mM, but very little for Na^+. The second carrier mechanism is relatively nonspecific for cations when K^+ is greater than 1 mM. In the latter case a cation like Na^+ is highly competitive with K^+ for absorption sites. Hanson and Kahn (18) also suggest a multiple carrier system for K^+ in corn roots.

Today it is possible to treat plants with inhibitors of protein synthesis to determine the relationship of protein synthesis to the rest of plant metabolism. The use and action of these inhibitors is of particular interest, since this thesis is concerned with the possible connections between protein synthesis and ion transport.

Rabinowitz et al. (41) suggest that amino acid analogues can block the incorporation of the natural amino acid and thereby inhibit protein synthesis. The literature concerning the uses of these analogues is voluminous. Hochster and Quastel (20) provide an excellent review of this subject. Ethionine (46),

4-aza-leucine (49) and 2-amino, 3-phenyl-butanoic acid (8) have all been demonstrated to inhibit bacterial protein synthesis. The use of D-serine by Ellis et al. (11) inhibited both protein synthesis and ion uptake in red beet root slices. The purine analogue 8-aza-guanine has also proved to be an excellent inhibitor of protein synthesis and growth in isolated root tips of peas (19).

The use of antibiotics in protein synthesis inhibition studies in plant tissue is a useful tool. Pathier (36) has recently reported the effectiveness of such specific inhibitors as chloramphenicol, puromycin, and erythromycin in retarding protein synthesis in tobacco leaf tissue. Chloramphenicol has been particularly effective in suppressing protein synthesis in a variety of plant physiological experiments (27, 35, 36, 38, 48).

The action of chloramphenicol has been the subject of intensive research in recent years, with almost all of the work being done on bacterial systems. Brock (5) published a very excellent review in 1961 on the use of chloramphenicol. He concluded that no accurate experimental evidence had been given showing that chloramphenicol had any direct effect on respiration. He criticised Kushner (30) for growing Pseudomonas on one substrate and then giving the bacteria a different substrate for the experimental period when he was testing the effect of chloramphenicol on respiration. Instead of inhibiting respiration, Brock suggested that Kushner had actually inhibited the enzymatic adaptation mechanism of the bacteria.

Gale (15) has presented an outstanding review on the action of many of the important antibiotics that are used today. While explaining the various mechanisms of specific inhibitors of protein synthesis, he describes the latest known biosynthetic pathway for protein synthesis. Gale further reported that chloramphenicol totally inhibits protein synthesis; on the other hand, it has very little effect on deoxyribonucleic acid (DNA), stimulates ribonucleic acid (RNA) turnover but not net RNA, and does not alter the properties of the membrane wall. The actual site of action of chloramphenicol is now thought to be the ribosome (5, 15, 24, 60, 64). Wolfe and Hahn (66) have recently proposed that chloramphenicol is binding at a 1:1 ratio to the ribosomes and thereby interferes with the function of messenger RNA.

The action of puromycin is somewhat similar to that of chloramphenicol (15) but instead of the antibiotic remaining bound to the ribosome, it strips off unfinished peptide chains from the ribosome (64).

Actinomycin-D is presently considered a specific inhibitor of DNA dependent RNA synthesis (15). This inhibitor, like chloramphenicol, is not supposed to effect the mucopeptides in the membrane wall.

Several ion transport mechanisms appear to be operating in the plant at the same time. Active rather than passive uptake is likely the more important means of ion accumulation in plants since greater ion concentrations are found within the plant than occur in the growth medium. It also appears

that some type of carrier within the plant cell selectively helps to transport entering ions across the cell permeation barrier. The use of specific inhibitors of protein synthesis has demonstrated that there is some connection between protein synthesis and ion absorption. Unfortunately, no data is available comparing the metabolic rate of protein synthesis to that of ion transport. The results reported here elaborate on the relationships between an inhibitor of protein synthesis, and protein synthesis and ion uptake on a kinetic basis.

METHODS AND MATERIALS

Euglena gracillis var. bacillaris was used as the test organism in this study. The strain was received as a gift from Dr. J. A. Shiff, Brandeis University, Waltham, Massachusetts. The benefits of using such an organism for a nutritional study have already been suggested by Sutcliffe (57), Price and Vallee (40), and Smillie (47, 48). Some of the reasons for using a unicellular algae instead of plant tissue include the following: a more rapid metabolism, a wider range of experimental conditions that the cells can tolerate, and the relative ease of obtaining uniform cell suspensions from day to day.

Euglena gracillis var. bacillaris is a unicellular chlorophyllous protozoan, better termed an algal flagellate. It is considered a plant when grown in the light on CO_2-enriched air in the presence of vitamins B_1 and B_{12}. Cells grown in this way would be termed autotrophic as opposed to heterotrophically grown cells that are cultured on carbon chain substrates.

The unicellular protozoan has been described by Wolken (67) as measuring about 50 μ x 15 μ when the alga is in its active condition. The cellular structure has a normal cell membrane that is covered with an exoskeleton called the pellicle. The pellicle is a membraneous structure, about

0.1-0.25 μ thick, composed of a system of semi-rigid rings alternating with strips of pliable membrane. The ultrastructure of the pellicle complex has recently been detailed by Sommer (50), indicating that the pellicle is continuous around the entire cell.

Method of Culture

Euglena was maintained on agar slants that consisted of 10 ml of the nutrient solution listed in Table 1, plus 5 per cent agar and 0.05 per cent Proteose Peptone (Difco-Fisher Scientific Company). Maximum precautions were taken to assure the purity of the strain. The cells were grown on slants for three weeks before one inoculating needle of cells was transferred aseptically to 1 liter of nutrient solution (as listed in Table 1) in a 2⅓ liter Fernbach culture flask. The flask was fitted with a glass tube bent so that it had one of its ends opening horizontally below the solution level near the edge of the flask. Air enriched to about 5 per cent carbon dioxide was pumped through the tube. This method provided a desirable level of carbon dioxide for autotrophic growth as well as a means of constant agitation of the cell suspension. The air mixture was bubbled through water to saturate it in order to reduce evaporation of the nutrient solution. The gas was then pumped through a DA-Millipore filter (0.65) to minimize the chances of bacterial contamination. Finally, the gas passed over a small water trap to remove any excess water before it entered the growth flask.

TABLE 1. Nutrient Solution Composition

Ion	Concentration
Macronutrients	
NH_4^+	2.0 m equiv./l
K^+	1.0 m equiv./l
Mg^{++}	1.0 m equiv./l
Ca^{++}	1.0 m equiv./l
$H_2PO_4^=$	0.5 m equiv./l
$SO_4^=$	3.0 m equiv./l
Cl^-	1.0 m equiv./l
NO_3^-	1.0 m equiv./l
Micronutrients	
Fe^{+++} (Sequestrene Na Fe)	0.50 ppm
$MnCl_2$	0.90 ppm
$ZnSO_4$	0.11 ppm
$CuSO_4$	0.04 ppm
H_3BO_3	1.43 ppm
H_2MoO_4	0.01 ppm
Vitamins	
B_1 (Thiamine-HCl)	0.06 g/l
B_{12} (Cyanocobolamin)	10.00 g/l

A second glass tube, fitted with a large cotton plug, was also inserted into the rubber stopper of the flask to serve as an outlet for the flow of gas.

After inoculation, the flask was placed in an incubator at 25±1°C in continuous light at 500 foot candles. The cells were harvested after six days. Usually the cells in culture solution were harvested in duplicate; the contents of three pairs of flasks could be harvested per week. Between cultures all flasks were washed for one-half hour with a mixture of hot nitric and sulfuric acids. They were then carefully washed and rinsed with distilled water. Standard procedure also included autoclaving the growth flasks, previously fitted with the aerating tube and filled with the nutrient solution.

Method of Harvesting

Cells were always harvested during their logarithmic division stage. They were collected in 250 ml polycarbonate centrifuge containers by centrifugation at 2000 x g for ten minutes. The supernatant fluid was carefully decanted, and the top layer of sediment was washed off and also decanted. This last step was done as an additional precaution to remove any possible contaminating bacteria which might have been present. The remaining sedimented cells were then washed with 250 ml of distilled water and recollected as mentioned above. The algae were then resuspended in distilled water and diluted to a known suspension density. The Klett-Summerson

colorimeter using a green filter (#54), as reported by Price and Vallee (40) and Klein (29), proved to be a very reliable means of making reproducible suspensions from day to day. Usually the dry weight never varied more than ±1 mg between treatment flasks for any experimental day. Suspensions were usually about 15 mg dry wt./100 ml of solution, but sometimes this had to be varied from a low of 10 mg to a high of 25 mg dry wt./100 ml.

Method of Treatments:

Standard Procedures

The cell suspensions were evenly distributed into 100 ml volumetric flasks. Unless otherwise noted, the flasks also contained a substrate and an inhibitor whenever used. After being made to volume, suspensions were transferred to 250 ml polycarbonate centrifuge containers and placed in a Warburg water bath at 25±0.1°C. The experimental flasks were lighted (1400 foot candles) from below unless otherwise noted. The containers were continuously aerated by pumping water-saturated air through ¼-inch diameter wooden dowels that were inserted into rubber tubing. The aerators were used to maintain constant agitation as well as to provide a desirable (62) supply of oxygen to the algae. It was found that autoclaving the cut dowels in distilled water for one-half hour opened most of the pores of the wood.

The cell suspensions were allowed to preincubate 1 hour before Rb^+ was added, in order to permit the cells to adjust

to the experimental conditions. Rubidium chloride (RbCl), at a concentration of 1 mM, was used as the tracer cation since it did not occur in the cells or growth medium. The use of Rb^+, at the above concentration, has been demonstrated by many workers (28, 33, 43) as an effective substitute for K^+.

Treatments were rapidly stopped by collecting the cells by centrifugation in the same manner as was employed for harvesting. The cells were washed before they were quantitatively transferred into tared 50 ml beakers and dried in an oven at 70°C.

After determining the net weight of the dried cells, they were digested by a nitric acid-hydrogen peroxide method. The remaining ash was dissolved in 1 N HCl and made up to a standard volume in the presence of excess K^+ at 375 ppm. Rb^+ concentrations were measured with a Perkin Elmer Atomic Absorption Spectrophometer (model No. 214). Rb^+ absorbed into the algal cells was expressed on a dry weight basis.

Experiments to Determine Treatment Parameters

Cells were cultured on the inorganic nutrient medium listed in Table 1, but Proteose Peptone was used as the carbon source in these preliminary studies instead of CO_2-enriched air. No carbon substrate was added during the Rb^+ absorption period which was 24 hours.

In order to determine the importance of ions bound to the membrane by electrostatic charges, six flasks of cultured

organisms were allowed to take up Rb^+ for a 24 hour period. After the cells were collected and washed once with 100 ml distilled water, as previously described, three of the flasks were washed with 1 mM KCl while the cells in the remaining flasks were washed a second time with distilled water. This same experiment was repeated but, this time, cells in half of the flasks were washed with 1 mM HCl. The amounts of Rb^+ absorbed are given in Table 2.

One 24 hour absorption experiment was performed in a citric acid-Na_2HPO_4 buffer solution (16) at pH 6.0, pH 7.0, and pH 8.0 to determine the effect of pH on the Rb^+ uptake. Each treatment was duplicated. On a second day the same type of uptake study was made in the presence of 50 mM Tris (hydroxymethyl) aminomethane HCl buffer at pH 7.2, pH 7.7, and pH 8.1. The mean Rb^+ accumulations are given in Table 3.

Time Studies

Treatments were initiated as detailed in the general methods, using 20 mM of ammonium glutamate (L-glutamic acid neutralized with concentrated NH_4OH and adjusted to pH 6.1 with HCl) as both a substrate (1) and a buffer. The treatments were run in replicates of three in this and succeeding experiments unless otherwise noted. Sample times included ½, 1, 2, 4, and 10 hours for those cells in the light and 1, 2, and 6 hours for those in the dark. These results are shown in Table 4.

The next experiment in the time study series was performed in the dark at 1°C in order to minimize the effect of active transport on total ion accumulation. At the same time, a parallel uptake series was performed similar to the first time study. The test times included 15 and 30 minutes for both experiments and 1, 2, and 6 hours for the cell suspensions at 25°C. A final time study was made at 1°C with samples taken at 2, 4, and 6 hours. The data for this group of experiments is in Table 4.

Experiments to Determine Characteristics of Active Uptake

Since it was desirable to determine the Q_{10} of the uptake process, but because the laboratory did not have a lighted refrigerated bath, it was first necessary to find out if light had any effect on low temperature ion uptake. Thus an experiment was conducted in both the light and the dark in the presence of ammonium glutatamate (20 mM) at 5°C for 1 and 2 hours. The results of this test are shown in Table 5. For this experiment ice cold water was circulated between the refrigerated water bath and the Warburg water bath.

The Q_{10} was then determined by simultaneously studying reaction rates in cell suspensions at 25°C and at 15°C in the dark. The treatment time was 1 hour, and all of the flasks containing 20 mM ammonium-glutatamate were in the dark for the entire period. The results are given in Table 6.

The action of 2, 4-dinitrophenol (DNP) on Rb^+ absorption was measured by comparing the effect of cell suspensions

treated with 1×10^{-5} or 1×10^{-4} M DNP to that of control conditions. No carbon substrate was used in this experiment but the pH was carefully monitored and maintained at 6.1 for the entire 1-hour uptake period. Treatments were duplicated. The data from this experiment is recorded in Table 7.

Effects of Carbon Substrates on Rb^+ Uptake

Several substrates, acetate, succinate, and ethanol, were studied to see if they affected Rb^+ uptake. These experiments were part of the preliminary ones that used the cells grown on the Proteose Peptone solution previously described. The acid form of both the acetate and the succinate was used instead of their salts, thus limiting the addition of any competing cations. The two concentrations used for the acids were 1 mM and 10 mM, while twice these amounts were used for the ethanol treatments. All treatments and controls were duplicated. The experimental data are given in Table 8.

Another experiment was designed to establish if the period of preincubation time with 20 mM ammonium glutamate had any effect on the amount of Rb^+ taken up by the algae in the following 2-hour absorption period. The cells were autotrophically cultured. The preincubation times were $\frac{1}{2}$, 1, 2, and 3 hours. The treatments were replicated three times and the results are given in Table 9.

Effect of Amino Acid Analogues on Rb^+ Uptake

The following amino acid analogues (one per experiment) were all used at 0.1 mM and 1 mM: D-leucine, D-serine,

DL-para-fluorophenylalanine, and 2-amino, 3-phenyl-butanoic acid. Each treatment was duplicated and two controls were included in every experiment. The uptake time was 1 hour following the normal 1 hour preincubation time. No substrate or buffer was used, but the pH was monitored before and after the absorption period. Similar experiments were used for ethionine, 4-aza-leucine-HCl, D-Serine, and 7-aza-tryptophane, except the uptake period was extended to 6 hours, 2 hours, 3½ hours, and 3½ hours, respectively. At the end of all these experiments, a 3 ml portion of the cell suspensions was removed and tested for net protein, by the method of Bostian and Price (3) which will be further explained in the last part of this section. These results are given in Table 10.

Effect of Specific Inhibitors of Protein Synthesis on Rb^+ Uptake

The following specific inhibitors of protein synthesis were studied: actinomycin-D at 1, 10, 20, and 40 μg/ml; puromycin at 5 and 10 μg/ml; ribonuclease at 10 and 25 μg/ml; and chloramphenicol (with and without the ammonium glutamate at 20 mM) at 0.25, 0.50, 0.75, 1.00, and 1.50 mg/ml. All the chloramphenicol used was its commercially available sodium succinate salt, which was highly soluble in water. Ammonium glutamate at 20 mM was used as a substrate. Preincubation time was 1 hour, followed by a 1 hour uptake period. All experiments were duplicated. Net protein in a 3 ml aliquot was also determined. The results from these tests are given in Tables 11 and 12.

In the first of the next series of experiments, cell suspensions were preincubated 2 hours with glycine-1-C^{14} (1.2×10^6 cpm, specific activity of 4.9 mc/mM) followed by an additional 2 hours of incubating with 0.50 mg/mg (1.5 mM chloramphenicol). Rb^+ was added at zero hour and the experimental flasks were removed at the end of 2, 3, and 4 hours. The treatments were run in replicates of three with an equal number of controls for each hour. The ammonium-glutamate substrate at 20 mM was present from the beginning of the experiment. These experiments were conducted in the light. Protein samples were taken from each flask before the cells were collected for Rb^+ analysis. The entire experiment was repeated a second time, but the ammonium glutamate, at the same concentration, was added at the same time as the Rb^+ was added (zero hour). A protein determination was made at zero hour as well as at the times when the flasks were removed for Rb^+ samples, which were 2, 4, and 6 hours. Finally, the last experiment was also repeated, except that no ammonium-glutamate was added; the pH of the solutions was adjusted to pH 6.1 with dilute NH_4OH and HCl. Also, sodium succinate at 1.5 mM was added to the control flasks at the same concentration present in the carrier salt of the chloramphenicol.

In order to determine the amount of bacterial contamination after a long experiment, a 1 ml aliquot was removed from a cell suspension from the above experiment after the completion of a 6 hour treatment (plus 2 hours preincubation)

and was serially diluted using aseptic procedures. The samples were then plated in Petri dishes containing 1.5 per cent agar and 1.5 per cent sucrose and the nutrient solution listed in Table 1, except for vitamin B_{12} which was omitted to stop the growth of the Euglena.

The specific activity of the protein samples was determined by first placing a 2 ml cell suspension on a DA-Millipore filter and washing it with 10 ml of distilled water. The cells were then washed with 15 ml of 6 per cent TCA for 1 minute, followed by two rapid washing with the same amount of TCA. The protein was dissolved off the filter paper into a 5 ml volumetric as described by Bostian and Price (3), and Folin Ciocalteau phenol reagent was added to measure the amount of protein present in the 5 ml of recovered solution. The optical density was read on a Klett-Summerson Colorimeter using a red (#66) filter as recommended by Lowry et al. (31). Having determined the amount of protein present, 20 ml of 15 per cent TCA was added to the alkaline solution containing the protein in a 50 ml Erlenmyer flask. This solution was poured through a second DA-Millipore filter and the Erlenmyer flask was rinsed once with 5 ml of 20 per cent TCA followed by a final rinse of 10 ml of distilled water. The Millipore filters were secured to aluminum planchets with rubber cement and dried on a warm hotplate. Cells from a 1 ml portion of algae suspension were also collected on a third Millipor filter and washed with distilled water (three 10 ml washings). This last

sample was to determine the total C^{14} uptake by the cell. All three samples (three from each treatment flask) were counted for radioactivity with a Nuclear Chicago Automatic Micro-Mil Window gas flow counter. All samples were counted for at least 10 minutes or 10,000 total counts and all were corrected for background which was never above 17 cpm. The C^{14} incorporation into protein was expressed as cpm/ g of protein. Because the amount of glycine uptake varied, incorporation was corrected proportionally to the amount of uptake. This was done by taking the reciprocal of the average of the net amount of total uptake of C^{14} into the cell for a particular sampling hour and multiplying it times the absolute specific activity of the protein. The results of these experiments are given in Table 14.

Source of Special Reagents

All amino acid analogues, as well as the puromycin and ribonuclease, were obtained from the Nutritional Biochemical Company. Chloramphenicol was purchased as the commercially available drug Chloromycetin from Parke Davis Company. Actinomycin-D was a gift of Merke, Sharpe, and Dohme. The glycine-1-C^{14} was purchased from New England Nuclear Company.

RESULTS

Experiments to Determine Treatment Parameters

Washing the cells with either 1 mM KCl or 1 mM HCl had no significant effect on removing bound Rb^+ from the cells, as shown in Table 2. These results demonstrate that the Rb^+ remaining in the algae after having been washed once with distilled water is primarily bound or incorporated Rb^+, not in a fraction that could be considered free space.

It is interesting to note the vast difference in the amount of uptake between the two experiments. Unfortunately, no particular reason can account for this except that this experiment was a preliminary one, and considerable variation was recorded in many of the early studies. Actually, the amount of 9.00 μM Rb^+/100 mg dry weight is quite close to the average amount of Rb^+ taken up by the algae in non-buffered solutions free of any carbon substrate. The important point is that for the experiment in question the actual amount of Rb^+ that was found in the cell had no real bearing on the determination of where the Rb^+ was bound.

TABLE 2. Effect of washing with KCl and HCl on accumulated Rb^+ by Euglena in 24 hours (μM Rb^+/100 mg dry wt.)

	Distilled water	1 mM KCl	1 mM HCl
Experiment 1	5.14[a]	5.38[a]	....
Experiment 2	14.85[b]	....	14.86[b]

Means not followed by the same letter are significantly different at the 5 per cent level (7).

The results of the pH study (Table 3) indicate that Rb^+ accumulation by Euglena has a definite sensitivity to pH. The data show that from pH 6.0 to pH 7.2 there was no significant effect on Rb^+ uptake, but as the pH is increased, Rb^+ absorption is distinctly stimulated. This effect of pH is quite similar to the results reported by Hurd and Sutcliffe (22).

Although increasing the pH from 6.0 to 8.0 was effective in stimulating the amount of Rb^+, the total amount of Rb^+ accumulated was less than the amount absorbed when no buffer at all was used for a 24 hour experimental period. It is quite possible that some of the cations contained in the buffers were competing with Rb^+ and thereby reduced the net uptake of Rb^+. For this reason it was decided that it would be desirable to avoid using a buffered system for the ensuing experiments. It was found that adding ammonium glutamate at a concentration of 20 mM at pH 6.1 served to hold the pH almost constant for as long as a 10 hour treatment. Actually, in the last experiment performed it was learned that simply adjusting the pH to 6.1 with dilute NH_4OH or HCl was all that was necessary to maintain a constant pH over a period of 8 hours. The reason for using pH 6.1 was that even if the pH did vary slightly, it would be unlikely to have any significant effect on the amount of Rb^+ uptake. Also, it was found that the pH of a non-buffered system adjusted to pH 7.5 would drift downward in the space of several hours.

TABLE 3. Effect of pH on Rb^+ uptake by Euglena in 24 hours (μM/100 mg dry wt.)

Buffer	pH 6.0	7.0	7.2	7.7	8.0	8.1
Citric Acid	3.12[a]	3.81[a]	....	....	7.66[c]	....
Tris	....	....	4.53[a]	6.28[b]	....	8.17[c]

Means not followed by the same letter are significantly different at the 5 per cent level (7).

Time Studies

The results given in Table 4 and schematically shown in Figure 2 demonstrate that Euglena in the light at 25°C rapidly took up Rb^+; after about an hour the rate decreased to a steady level for five hours more before leveling off. When this experiment was repeated on a different day, the same rapid accumulation of Rb^+ was found, but the rate of uptake leveled off significantly different from the rate of the first day's experiment. The algae absorbed almost as much Rb^+ in the dark at 25°C in a short period as they did in the light at the same temperature. As the uptake time increased, the amount of Rb^+ accumulated was significantly less in the dark. Even though the net amount of Rb^+ for cells in the dark was less than for those tested in the light, the rate of uptake (as shown in Figure 2) was quite similar for both groups of cells.

It was found that almost all of the Rb^+ that was going to enter the cell under these conditions did so in the first 15 minutes when cells in the dark at 1°C were supplied with Rb^+.

TABLE 4. Time study of Rb^+ uptake by Euglena (μM Rb^+/100 mg dry wt.)

Day	Conditions	Time-Hours ¼	½	1	2	4	6	10
1	25° light	....	1.88^{b}	3.87^{de}	5.18^{fg}	6.45^{hi}	8.05^{j}	8.77^{j}
	25° dark	....	....	3.27^{cd}	4.18^{e}	....	6.78^{i}	....
2	25° light	....	2.12^{b}	2.97^{c}	4.37^{ef}	....	5.65^{gh}	....
	1° dark	0.48^{a}	0.79^{a}	....	....	....	....	....
3	1° dark	....	....	....	0.41^{a}	0.57^{a}	0.57^{a}	....

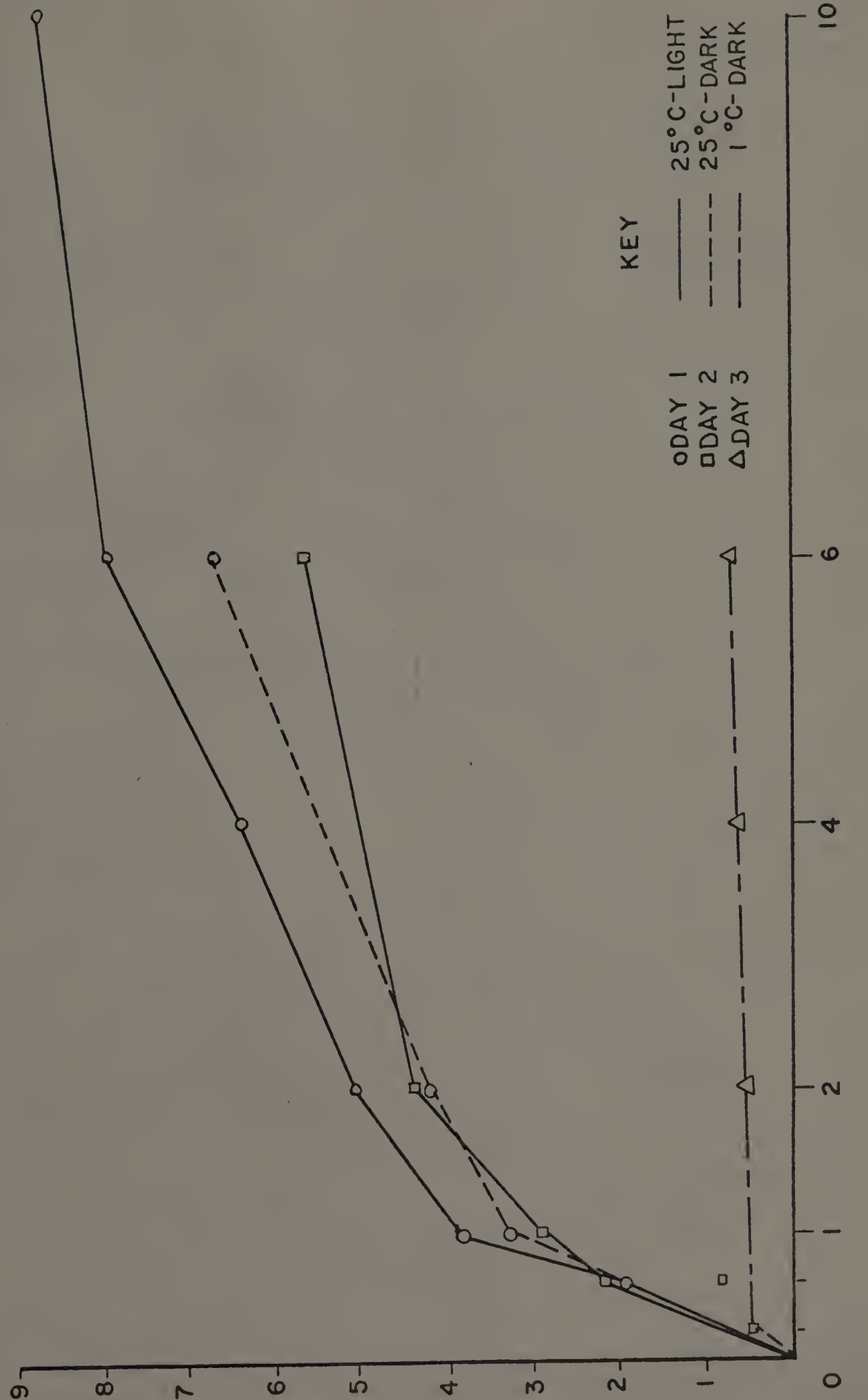
Means not followed by the same letter are significantly different at the 5 per cent level (7).

Experiments to Determine Characteristics of Active Uptake

The data shown in Table 5 indicate ther was no significant effect of light and dark on Rb^+ uptake when the absorption experiment was conducted at 5°C. The results of this test suggested that it was possible to use a second water bath that was unlighted for reduced temperature studies.

The Q_{10} of the uptake process was determined to be 1.89, as reported in Table 6. DNP was only effective in inhibiting Rb^+ uptake when used at 1.0×10^{-4} concentration. The data from this experiment are given in Table 7. These last two

Figure 2. Time study of Rb^+ uptake by Euglena.



experiments indicate that active uptake is occurring within 1 hour after the addition of Rb^+. It should, therefore, be possible to test the active uptake process in other experiments of similar duration.

TABLE 5. The effect of light on uptake of Rb^+ by Euglena at 5°C (μM Rb^+/100 mg dry wt.)

		Time-Hours 1	2
Light	5°C	0.76[a]	1.07[b]
Dark	5°C	0.77[a]	0.97[ab]

Means not followed by the same letter are significantly different at the 5 per cent level (7).

TABLE 6. The effect of temperature on Rb^+ uptake in 1 hour by Euglena in the dark (μM Rb^+/100 mg dry wt.)

25°C	15°C	Temperature Coefficient
1.95	1.03	1.89

TABLE 7. The effect of 2, 4-dinitrophenol (DNP) on Rb^+ uptake by Euglena for 1 hour (μM Rb^+/100 mg dry wt.)

	DNP Concentration	
Control	1×10^{-5}M	1×10^{-4}M
2.97[a]	2.97[a]	2.01[b]

Means not followed by the same letter are significantly different at the 5 per cent level (7).

Effect of Carbon Substrates on Rb^+ Uptake

The addition of key metabolic intermediates at concentrations ranging from 1 mM to 2 mM significantly stimulated

Rb^+ absorption (Table 8). When either of these substrate concentrations of succinate or ethanol was increased 10 times, there was no significant increase in Rb^+ uptake as compared with the controls. Acetate at 10 mM actually proved to be lethal to the cells.

TABLE 8. The effect of carbon substrates on Rb^+ uptake by *Euglena* for 24 hours (μM Rb^+/100 mg dry wt.)

	None	1 mM	2 mM	10 mM	20 mM
Acetate	6.75^{b}	13.69^{cd}	....	0.00^{a}	....
Succinate	8.20^{b}	15.74^{cd}	....	7.85^{b}	....
Ethanol	10.11^{bc}	....	14.98^{d}	....	6.60^{b}

Means not followed by the same letter are significantly different at the 5 per cent level (7).

Lengthening the preincubation time with 20 mM ammonium glutamate (see Table 9) had no significant effect on the net amount of Rb^+ absorbed (in a uniform uptake period) unless the preincubation time was longer than 2 hours. Although Rb^+ uptake did not significantly increase for each additional time interval tested, it should be noted that the net uptake did steadily rise.

TABLE 9. The effect of preincubation time with 20 mM ammonium glutamate on Rb^+ accumulation (μM Rb^+/100 mg dry wt.)

Time-Hours			
$\frac{1}{2}$	1	2	3
4.22^{a}	4.59^{ab}	5.12^{bc}	5.51^{c}

Means not followed by the same letter are significantly different at the 5 per cent level (7).

Effect of Amino Acid Analogues on pH and Rb^+ Uptake

There was no effect of the following amino acid analogues at either 0.1 mM or 1.0 mM on Rb^+ uptake in a 1 hour experiment: D-serine, DL-para-fluorophenylaline, D-leucine, and 2-amino, 3-phenyl butanoic acid, as shown in Table 10. The following analogues: D-serine, 7-aza-trytophane, and ethionine, used at the same concentrations as above for longer experimental periods still did not show any significant inhibition. The analogue 4-aza-leucine-HCl at 0.1 did significantly inhibit Rb^+ uptake after 1 hour, but at ten times this concentration there was no significant inhibition. No significant differences in the amount of net protein recovered from the sample aliquots was found in cells treated with these or the above-mentioned analogues. The amino acid analogues used in these experiments did not influence the amount of Rb^+ accumulated or the net protein content of the treated cells. It was, therefore, deemed desirable to investigate the effects of specific inhibitors of protein synthesis on the absorption of Rb^+.

Effect of Specific Inhibitors of Protein Synthesis on Rb^+ Uptake

When the specific inhibitors of protein synthesis, actinomycin-D, puromycin, and ribonuclease, were used at the respective concentrations listed in Table 11, no significant retardation of Rb^+ uptake was found. These inhibitors did not effect the net amount of protein recovered from the sample aliquots.

TABLE 10. Effect of amino acid analogues on pH and Rb^+ uptake by Euglena in 1 hour (μM Rb^+/100 mg dry wt.)

Analogue	Absorption Time-Hours	Concentration					
		None		0.1 mM		1.0 mM	
		pH	Rb^+	pH	Rb^+	pH	Rb^+
D-serine	1	6.5	2.72^{bcd}	6.5	2.84^{cd}	6.3	2.36^{abcd}
DL-para-fluoro-phenylalanine	1	5.6	1.85^{abc}	5.6	2.01^{abc}	5.6	1.14^{a}
D-leucine	1	5.6	1.41^{a}	5.6	1.54^{ab}	5.5	1.74^{abc}
2-amino, 3-phenyl butanoic acid	1	5.7	1.65^{abc}	4.4	2.15^{abcd}	3.3	1.97^{abc}
4-aza-leucine-HCl	2	5.2	3.31^{d}	4.6	1.80^{abc}	3.6	2.18^{abcd}
D-serine	3½	6.0	4.58^{e}	6.0	4.90^{e}	5.8	4.83^{e}
7-aza-tryptophane	3½	5.2	3.29^{d}	5.1	3.00^{cd}	5.6	3.26^{d}
ethionine	6	6.4	5.69^{ef}	6.3	6.13^{f}	6.0	6.48^{f}

Means not followed by the same letter are significantly different at the 5 per cent level (7).

TABLE 11. Effect of specific inhibitors of protein synthesis on Rb^+ uptake (μM Rb^+/100 mg dry wt.)

Inhibitor	Concentration - μg/ml						
	0	1	5	10	20	25	40
Actinomycin-D	1.35[a]	1.35[a]	....	1.11[a]	1.53[a]	....	1.46[a]
Puromycin	2.07[b]	....	2.32[b]	2.49[b]	....	....	....
Ribonuclease	1.07[a]	....	....	1.01[a]	....	1.20[a]	....

Means not followed by the same letter are significantly different at the 5 per cent level (7).

When chloramphenicol was used at concentrations ranging from 0.25 mg/ml to 1.50 mg/ml with and without ammonium glutamate (20 mM), it was found that concentrations of at least 0.75 mg/ml and 1.00 mg/ml of the antibiotic, respectively, were needed to significantly inhibit Rb^+ absorption within a 1 hour uptake period, providing that cells were preincubated for the same amount of time with the inhibitor. These results, as shown in Table 12, indicate that a greater inhibition occurred when the experiment was done in the presence of the ammonium glutamate at pH 6.1. Determinations of the net protein content in the aliquots of cells sampled showed no differences between any of the treatments. It, therefore, was apparent that measurements of the net protein content of the cells would not be sufficient to determine the effect of chloramphenicol on protein synthesis. Parthier (36) recently reported that he could not measure any differences in net

protein when he treated tobacco leaves with various antibiotics including chloramphenicol.

TABLE 12. Effect of chloramphenicol on Rb^+ uptake (μM Rb^+/100 mg dry wt.)

Substrate	Concentration-mg/ml					
	0	0.25	0.50	0.75	1.00	1.50
None	3.22^{b}	2.74^{ab}	2.69^{ab}	2.60^{ab}	1.74^{a}	.....
Ammonium - glutamate	4.35^{c}	4.25^{c}	....	3.50^{b}	3.15^{b}	2.33^{a}

Substrate means not followed by the same letter are significantly different at the 5 per cent level (7).

In the first experiment to determine the simultaneous rate of Rb^+ uptake and protein synthesis, the specific activity of the protein was very low and hardly increased during the treatment period. The net amount of Rb^+ absorbed was not inhibited at all until the last sampling time, which was 4 hours. It was noted that the ^{14}C content of the cells did not increase after the first set of cell suspensions were removed for Rb^+ determinations.

In view of the difficulties experienced in the above experiment, a second experiment was performed similar to the first except that a longer test period was used. This experiment demonstrated that the amount of Rb^+ uptake was further inhibited with increased time. This was checked for bacterial contamination, and it was found that the cell suspension had

less than 1 x 10^5 bacteria/ml after 8 hours. It was also learned that adding the ammonium glutamate at the same time the Rb^+ was added allowed a greater amount of ^{14}C-glycine to enter the cell. These results indicate that the ammonium glutamate which was several hundred times more concentrated than the ^{14}C-glycine was acting as a competitive inhibitor of the incorporation of labeled amino acid into protein. Unfortunately, the addition of the ammonium glutamate seemed to block the detection of any differences in the specific activity of the protein between the chloramphenicol treated and non-treated samples after the first protein sample was taken. Since it was apparent that there was a distinct difference in the specific activity between the protein samples before the ammonium glutamate was added, it seemed logical to attempt to run a similar experiment in the absence of ammonium glutamate.

In the last experiment, the pH was adjusted and maintained at 6.1 with NH_4OH and HCl, and the labeled glycine was added ½ hour before the Rb^+ was added (1½ hours after the chloramphenicol was added). The results for the amounts and rates of Rb^+ uptake were quite similar to those of the last experiment. The mean values for each treatment (averages from the two experiments) are given in Table 13. The determinations for the specific activity of the protein did show continuous increases throughout the test period and significant

differences were observed between the chloramphenicol treated and non-treated systems. These data are given in Table 13.

The data in Table 13, as schematically shown in Figure 3, have particular importance when protein synthesis inhibition is compared with the rate of inhibition of Rb^+ uptake. The rate of protein synthesis was reduced 2 hours sooner than the rate of Rb^+ accumulation. It also should be noted that even though the rate of protein synthesis in the chloramphenicol treated system was still proceding at 60 per cent of the rate of the cells without chloramphenicol, the rate of Rb^+ transport in the inhibited system was zero. These data indicate a very likely relationship between protein synthesis and Rb^+ accumulation. It is quite possible that the antibiotic is slowly reducing the amount of the hypothetical ion-carrier molecule which could be just one protein of the many hundreds present in the algae.

TABLE 13. Effect of 0.50 mg/ml chloramphenicol on ^{14}C incorporation into protein[1] (cpm/µg protein) and Rb^+ uptake[2] on a time basis (µM Rb^+/100 mg dry wt.)

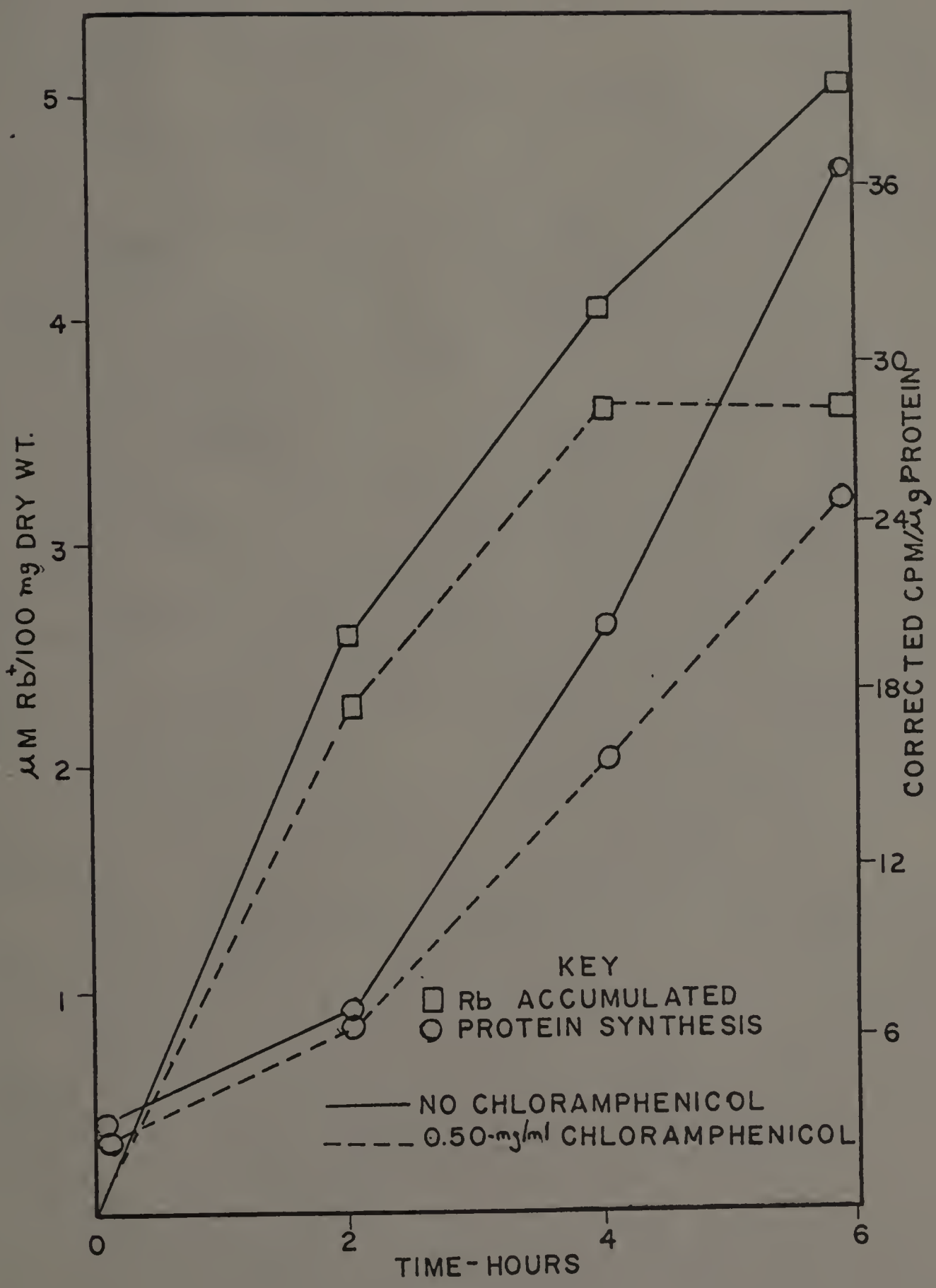
Time-Hours		Control	Chloramphenicol	Per Cent Inhibition
0	cpm/µg protein	3.19[a]	2.81[a]	15
2	cpm/µg protein	7.43[b]	6.90[ab]	7
	Δ cpm/µg protein	4.24	4.09	4
	Rb^+	2.58[g]	2.34[g]	9
	Δ Rb^+	2.58	2.34	9
4	cpm/µg protein	20.55[d]	15.75[c]	23
	Δ cpm/µg protein	13.12	8.85	32
	Rb^+	4.07[i]	3.69[h]	9
	Δ Rb^+	1.49	1.35	9
6	cpm/µg protein	37.31[f]	25.75[e]	31
	Δ cpm/µg protein	16.76	10.00	40
	Rb^+	5.11[j]	3.61[h]	29
	Δ Rb^+	1.04	-0.08	100

Means not followed by the same letter are significantly different at the 5 per cent level (7).

[1]Protein means represent 3 samples.

[2]Rb^+ means represent 6 samples.

Figure 3. Protein synthesis and Rb^+ uptake on a time basis.



DISCUSSION

The Rb^+ retained by Euglena cells after they were washed once with distilled water was held by fractions of the cell other than portions designated as free space. The results obtained indicate that this is likely to be the case since the first wash probably removed Rb^+ that was in water free space and the experimental wash with KCl or HCl should have released Rb^+ bound in Donnan free space. Since no differences were recorded following washing with water, KCl, or HCl, it can be assumed that Donnan free space is not an important consideration in Euglena.

The variability experienced in many of the preliminary experiments could be attributed to several causes. First, it is now known that the dry weight, protein and RNA content of Euglena steadily decreases with time as cells divide exponentially (65). Therefore, if cells were slightly older in one experiment than in another, their entire metabolic composition would be different. Second, the amount of Rb^+ accumulated might vary from day to day as the concentration of the ions already present in the cells varied. This variation could also be related to the age of the culture since there would be progressively more cells present in the culture. With a larger number of cells, all removing a portion of the remaining nutrient solution, a time is reached when ion availability is limiting. This state might result in reduced ion absorption per cell. Schaedle and Jacobson (45) have reported

that in Chlorella the initial amount of K^+ present in the cell had a distinct effect upon subsequent absorption of K^+. Finally, in a 24 hour uptake experiment, many difficulties could arise, such as bacterial contamination, exhaustion of substrates (particularly of endogenous food reserves when no carbon substrate was added), drifting of the pH in non-buffered systems, and changes in metabolic rate or pathway (14). For these reasons Epstein et al. (14) have recommended the use of short term experiments (10 to 40 minutes) for plant absorption studies. This fact was kept in mind when further experiments were designed, except that in these experiments it was necessary to use longer periods of time in order to allow for a measurable amount of Rb^+.

The results of the time study at 25°C in the light are quite consistent with those of other workers (28, 57). The initial rapid uptake of the Rb^+ was followed by a leveling off of the absorption rate which became non-significant after about 6 hours. The difference in the cellular uptake between the two experiments at 25°C in the light can only be explained by the possible variability in the algae from day to day as previously described. The uptake curve for cells in the dark at the same temperature was almost parallel to that for the cells in the light. This result indicates that even though the total amount of Rb^+ that was absorbed by these cells in the dark was less than for those treated in the light, there

were enough carbon substrates present (either as endogenous reserves or as available from the ammonium glutamate) to allow the dark treated cells to have a normal metabolism.

The absorption curve determined at 1°C in the dark represents passive uptake since all metabolic processes would virtually cease at that temperature, thereby stopping active uptake. These data suggest that passive uptake is of major concern only in regard to the total uptake process for a very short time (less than $\frac{1}{2}$ hour) in proportion to the entire uptake period. The time uptake curves comparing a monovalent ion accumulation at 25°C to that at 1°C are quite similar to the ones reported by Sutcliffe (58) in carrot root slices.

The Q_{10} of 1.89 for the uptake process of 1 mM Rb^+ is indicative of an active uptake process as reported by Sufi (55) who actually found the Q_{10} to be 1.79 for 1 mM Rb^+ into bean plants. Jyung et al. (28) determined the same Q_{10} for enzymatically isolated tobacco leaf cells. The low amount of inhibition (33 per cent) resulting from treatment with 1×10^{-4} M DNP is also very similar to the results obtained by Jyung et al. (28) with isolated leaf cells.

The stimulation of ion uptake by the addition of key metabolic intermediates was very effective at substrate concentrations (1-2 mM). Mitsui and Hirata (34) found the same results for K^+ uptake into etiolated rice seedlings,

except they did not find succinate to have a significant effect. When the ethanol and succinate concentrations were increased ten times, they had no effect on Rb^+ absorption by Euglena. A partial Crabtree effect (63) where high concentrations of substrates actually retard respiration might account for this result. The higher concentration of acetate was probably lethal because the acid lowered the pH below a tolerable minimum.

In the report by Mitsui and Hirata (34), it is indicated that glutamate was an efficient substrate for K^+ accumulation. Glutamate was only effective when used with the Euglena for periods longer than 2 hours. The steady enhancement of Rb^+ absorption could be attributed to either the action of the glutamate as a substrate or as a source of additional organic acids. A greater concentration of organic acids present in the cell would result in more cation uptake, and cell electrical neutrality could still be maintained.

The amino acid analogues did not have any significant effect on Rb^+ under the conditions of these experiments. On the other hand, Ellis et al. (11) have reported that D-serine, at the concentration used in this study, did inhibit K^+ uptake into red beet and carrot slices. The action of the analogue, 4-aza-leucine-HCl is somewhat questionable since it was ineffective as an inhibitor when its concentration was increased 10 times the level where it acted as an

inhibitor. It is not likely to be a pH effect, for the higher concentration of the analogue resulted in greater acidity. Further work with the amino acid analogues did not seem warranted since they are nonspecific in their action, and it would not have been possible to state that they were blocking just one metabolic process.

The lack of inhibition by actinomycin-D, puromycin, or ribonuclease when used in 1 hour experiments might simply be due to the fact that insufficient time was allowed for cell penetration. The same situation occurred at low concentrations of chloramphenicol which were used in short-term experiments. If chloramphenicol is typical of all these specific inhibitors used, then it might be possible to conclude that there is a lag of almost four hours before these antibiotics have any significant effect on the protein synthesis within the Euglena. Pramer (39) has reported that chloramphenicol enters the alga Nitella clavata by only simple diffusion. When he treated his cells with 0.40 mg/ml chloramphenicol at pH 6.0, it took over 6 hours for the internal concentration to equilibrate with the amount of antibiotic in the external solution. He found that after 2 hours only one-third of the inhibitor had been absorbed and after 4 hours slightly more than half had accumulated. The results of this study would suggest that chloramphenicol absorption by Euglena was also very slow since a lag of 4 hours

in protein synthesis inhibition after the cells were in the presence of chloramphenicol was noted. The effect of increasing the concentration of chloramphenicol did show significant inhibition of Rb^+ uptake within 1 hour if the cells were first preincubated 1 hour. This effect could also be attributed to the fact that chloramphenicol was entering the cell by simple diffusion, since increasing the concentration of the external solution will often result in a more rapid transfer of some of the solute to the other side of the permeation barrier. Nooden and Thimann (35) also relate findings of a lag of inhibition of chloramphenicol on protein synthesis in artichoke tuber sections to the possibility that the antibiotic penetrates plant tissue very slowly as compared to bacterial cells because of differences in membrane characteristics.

The simultaneous determinations of the rates of both protein synthesis and Rb^+ accumulation were successful refinements of many of the earlier experiments with chloramphenicol as reported by other workers (4, 9, 10, 23, 27, 36, 56) who only conducted their experiments for one length of time which was usually so long that no differences could be detected. By closely monitoring the two rates, it has been shown that there is a very definite lag between the inhibition of protein synthesis and the following slower inhibition of Rb^+ uptake. The accumulation of Rb^+ was eventually completely inhibited while protein synthesis

continued at 60 per cent of its normal rate. Therefore, it seems unlikely that total protein turnover is directly related to ion transport. However, it is quite possible, as suggested by Jacoby and Sutcliffe (23), that a particular protein or enzyme is acting as the carrier and a certain amount of it is present in an endogenous reserve, so that even if the rate of this protein being synthesized was reduced, there might be a lag before the endogenous supply of the carrier is depleted, resulting in retardation of ion transport. There is yet another very distinct possibility that because the inhibitor has reduced the rate of protein synthesis, the entire enzyme level present in Euglena decreases so that the total metabolic rate of the algae is depressed to the point where active uptake is affected.

The action of chloramphenicol has been considered non-specific to any particular type of protein by most researchers (5, 15, 60, 64), but recently Pogo and Pogo (38) reported that chloramphenicol preferentially inhibited plastid protein synthesis in Euglena gracillis Z strain. This finding suggests that possibly only this main group of proteins was significantly reduced and the proposed ion carrier was in the remaining group of proteins that were only partially affected by the inhibitor. The results of this study can neither support nor refute the possibility of specific types of proteins being inhibited by chloramphenicol.

Most plant physiologists who have studied the effect of chloramphenicol on ion transport have found no effect on

respiration (23, 27, 37, 56, 59). However, Hanson and Hodges (17) and Stoner et al. (53), in the same laboratory, have reported that chloramphenicol acts as an uncoupler of oxidative phosphorylation. It is quite possible that an adaptation mechanism (an enzymatically directed adjustment to a new substrate) was inhibited and not respiration, for they applied 0.6 mg/ml chloramphenicol to isolated maize mitochondria in the presence of a pyruvate-succinate substrate. It can, therefore, only be assumed that because most other workers did not affect respiration when they treated their plant tissue with often higher concentrations of chloramphenicol than used in this study, respiration was not significantly affected in these experiments.

In summary, it was found that Euglena gracilis var. bacillaris accumulates most of its Rb^+ by an active uptake process. It is assumed that a proteinacious carrier is serving as the means of transport for the Rb^+ across the permeation barrier since inhibition of protein synthesis results in decreased Rb^+ accumulation.

SUMMARY

Experiments were conducted to determine the nature of Rb^+ absorption by Euglena gracilis.

1. The net Rb^+ recovered by Euglena after washing with distilled water or KCl and HCl was determined to be bound or incorporated Rb^+ in non-free space. From these results it was assumed that Donnan free space was not an important consideration in Euglena.

2. The rate of Rb^+ absorption by Euglena was found to be very rapid for 1 hour and then leveled off for another 5 hours after which little further accumulation was detected.

3. Passive uptake was found to be of little importance after the first ½ hour of Rb^+ absorption.

4. Active uptake in Euglena could be successfully studied any time after the first hour of an absorption experiment when 1 mM Rb^+ was used as the test cation.

5. Certain carbon substrates significantly stimulated Rb^+ absorption in Euglena.

6. The amino acid analogues studied did not affect the net amount of protein or inhibit Rb^+ uptake in Euglena.

7. Inhibition of Rb^+ uptake in Euglena with actinomycin-D, puromycin, or ribonuclease was unsuccessful. This was probably due to too short an experimental period.

8. A lag period in response to chloramphenicol by Euglena was noted. This was probably due to its slow penetration of the alga cell.

9. Chloramphenicol inhibited protein synthesis more rapidly than it inhibited Rb^{+} absorption in Euglena.

10. It is postulated that a protein is part of the carrier mechanism for ion transport and that the endogenous reserves of that protein must first be reduced before any effect on the amount of ion absorbed can be detected.

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